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(57) Abstract

The present invention relates to improved stable oil in water emulsions having an oil droplet diameter of substantially 300–600nm comprising triglycerides, and their use as vaccine adjuvants.

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OIL IN WATER VACCINE COMPOSITIONS

The present invention relates to an improvement in an oil in water vaccine composition. In particular, the present invention relates to a vaccine adjuvant formulation based on oil in water emulsion comprising a metabolisable oil squalene, α-tocopherol, TWEEN 80, which has been improved by the inclusion of a triglyceride, further, these vaccine adjuvants can optionally comprise an immunologically active fraction of QuilA (preferably QS21) and 3D-MPL.

Tricaprylin ($C_{27}H_{50}O_6$) is an oil of the tryglyceride family, and is known in the art (The Lipid Handbook (1986) Eds. Gunstone, F., Harwood, J. and Padley, F. Published by Chapman and Hall, pages 368-377).

QS21 is a HPLC purified non-toxic fraction of a saponin from the bark of the South American tree Quillaja Saponaria Molina, and the method of its production is disclosed (also known as QA21) in the US patent No. 5,057,540.

3 De-O-acylated monophosphoryl lipid A is a well known adjuvant manufactured by Ribi Immunochem, Montana. Chemically it is supplied as a mixture of 3 De-O-acylated monophosphoryl lipid A with either 4, 5, or 6 acylated chains. Alternatively, it can be manufactured according to the disclosure of GB 2,220,211 (Ribi). A preferred form of 3 De-O-acylated monophosphoryl lipid A and its method of manufacture is disclosed in International Patent Application No. 92/116556.

Oil in water emulsions *per se* are well known in the art, and have been suggested to be useful as adjuvant compositions (EPO 399843).

International patent application No.WO 95/17210 discloses an emulsion system based on squalene, α-tocopherol, and TWEEN 80, optionally formulated with the immunostimulants QS21 and/or 3D-MPL. These are very potent inducers of a wide range of immune responses, including Cytotoxic T-cell responses in some model systems. Induction of CTL responses occurs naturally during infection of a target cell, or uncontrolled synthesis of a tumour antigen, wherein enzymatic degradation of the target antigen takes place in the cell cytoplasm. This phenomenon allows cytoplasmic peptides

derived from the pathogen, or tumour specific antigen, to enter the Th1 pathway and be presented on the surface of the cell associated with an MHC class 1 molecule. If a vaccine antigen does not enter into, and replicate within, the cytoplasm of the host cell, then it will enter the Th2 pathway and ultimately be presented on the surface of the cell associated with a MHC class II molecule. This alternative route generally results in T-helper responses and antigen specific antibody responses.

As mentioned above, after vaccination a pathogen specific antigen does not enter the cytoplasm of a host cell, and therefore will not enter the Th1 pathway and ultimately induce a CTL response. A recognised signal that a Th1 response has been stimulated is the enhanced production of Th1-type cytokines *eg.* IFN-γ and IL-2. IFN-γ secretion is associated with protective responses against intracellular pathogens, including parasites, bacteria and viruses. Activation of leucocytes by IFN-γ enhances killing of intracellular pathogens and increases expression of Fc receptors. Direct cytotoxicity may also occur, especially in synergism with lymphotoxin (another product of TH1 cells). IFN-γ is also both an inducer and a product of NK cells, which are major innate effectors of protection. TH1 type responses, either through IFN-γ or other mechanisms, provide preferential help for murine IgG2a immunoglobulin isotypes.

The oil in water emulsions mentioned in the patent application above (International patent application No.WO 95/17210) when formulated with 3D-MPL and QS21 are potent inducers of Th1 type immune responses. Accordingly, these adjuvants when associated with antigen preferentially stimulate the sub-isotype of IgG associated with Th1 responses (murine IgG2a) and also will induce significant levels of IFN-γ production and antigen specific cytotoxic T lymphocytes (CTL) responses.

The observation that the basic oil in water/QS21/3D-MPL formulation can induce strong cytolytic T lymphocyte responses is significant, as these responses in certain animal models have been shown to induce protection against disease.

The examples described in the abovementioned International patent application No.WO 95/17210, compared various methods of formulation which resulted in oil droplets of different diameter. Preparations having a particle diameter of around 500nm, as

measured by photon correlation spectroscopy (mass distribution), showed superior adjuvant properties. This work highlighted the great benefit to be derived from the adjuvant with a larger sized oil droplet, in terms of increased IgG2a/b antibody titres and cell mediated immunity (CMI).

The emulsions of International patent application No.WO 95/17210, the use of which has been discussed above, obviously holds great advantages over conventional non-Th1 inducing adjuvants. Despite the fact that the larger particle stands out amongst the preparations as being the best size of droplet to induce immune responses, it suffers from the disadvantage of lack of stability. Unfortunately, because of the large size of the oil droplets in the emulsion, whilst being beneficial for the induction of immune responses, the emulsion breaks down. Indeed, in order to attain a uniform vaccine preparation, the emulsion has to be produced immediately prior to use.

The present invention solves the problem of emulsion instability and retains the preferred diameter of the large oil in water droplets for optimal adjuvanticity. This has been achieved by the formulation of the oil in water emulsion in the presence of a triglyceride oil.

The oil in water emulsions of the present invention are stable. Thus the oil droplet diameter remains at a relatively constant level over a prolonged period of time. Preferably the emulsions of the present invention will not cream, or separate into two phases, for a period of over 1 year at 4°C, and most preferably over 2 years.

Accordingly, in one preferred embodiment of the present invention provides a vaccine or pharmaceutical formulation comprising an antigen in conjunction with 3 De-O-acylated monophosphoryl lipid A, QS21, a triglyceride and an oil in water emulsion, wherein the triglyceride may be tricaprylin and the oil in water emulsion comprises a metabolisible oil (such as squalene), α-tocopherol and TWEEN 80. Such a formulation is suitable for a broad range of monovalent or polyvalent vaccines. Additionally the oil in water emulsion may contain span 85. A preferred form of 3 De-O-acylated monophosphoryl lipid A is disclosed in International patent application published under No. 92116556 - SmithKline Beecham Biologicals s.a.

The size range of oil droplet found within the stable oil in water emulsion is the important aspect of this invention. It is envisaged that embodiments of this invention will be in the range of substantially 300-600nm, preferably substantially around 350-550nm in diameter, and most preferably substantially 450-500nm in diameter as measured by photon correlation spectroscopy. Although many of the examples described herein have droplet sizes of substantially 500nm it will be appreciated by the man skilled in the art that the invention is applicable to oil in water emulsions wherein the oil droplets are greater than substantially 500nm in diameter, thus oil droplets may have an average droplet size of 600nm or greater. Accordingly, the oil droplet diameter may be within the range of substantially 500-600nm. The definition of substantially in relation to this invention is greater than 80% of the oil droplets by number being within the stated ranges, preferably greater than 90%, and most preferably greater than 95%.

In order for any oil in water composition to be suitable for human administration, the oil phase of the emulsion system has to comprise a metabolisable oil. The meaning of the term metabolisable oil is well known in the art. Metabolisable can be defined as "being capable of being transformed by metabolism" (Dorland's Illustrated Medical Dictionary, W.B. Sanders Company, 25th edition (1974)). The oil may be any vegetable oil, fish oil, animal oil or synthetic oil, which is not toxic to the recipient and is capable of being transformed by metabolism. Nuts, seeds, and grains are common sources of vegetable oils. Synthetic oils are also part of this invention and can include commercially available oils such as NEOBEE® and others. Squalene (2,6,10,15,19,23-Hexamethyl-2,6,10,14,18,22-tetracosahexaene) is an unsaturated oil which is found in large quantities in shark-liver oil, and in lower quantities in olive oil, wheat germ oil, rice bran oil, and yeast, and is a particularly preferred oil for use in this invention. Squalene is a metabolisable oil virtue of the fact that it is an intermediate in the biosynthesis of cholesterol (Merck index, 10th Edition, entry no.8619).

The oil in water emulsion may be utilised on its own or with other adjuvants or immuno-stimulants and therefore an important embodiment of the invention is an oil in water formulation comprising squalene or another metabolisable oil, a triglyceride, such as

tricaprylin, α-tocopherol, and polyoxyethylene sorbitan monooleate (TWEEN 80™). The oil in water emulsion may also contain span 85 and/or Lecithin. Thus, in one particular embodiment the present invention comprises an adjuvant composition comprising an oil in water emulsion consisting of squalene, TWEEN80™, α-tocopherol, wherein the oil droplets within the emulsion have an average diameter of substantially 300-600nm. The present invention also provides for a vaccine comprising an oil in water emulsion consisting of squalene, TWEEN80™, α-tocopherol, wherein the oil droplets within the emulsion have an average diameter of substantially 300-600nm, and an antigen or antigenic preparation. In another preferred embodiment, the adjuvant composition, or vaccine composition, as described above, further comprises QS21 and 3D-MPL.

Preferably the vaccine formulations of the present invention contain an antigen or antigenic composition capable of eliciting an immune response against a human pathogen, which antigen or antigenic composition is derived from HIV-1, (such as tat, nef, gp120 or gp160), human herpes viruses, such as gD or derivatives thereof or Immediate Early protein such as ICP27 from HSV1 or HSV2, cytomegalovirus ((esp Human)(such as gB or derivatives thereof), Rotavirus (including live-attenuated viruses), Epstein Barr virus (such as gp350 or derivatives thereof), Varicella Zoster Virus (such as gpI, II and IE63), or from a hepatitis virus such as hepatitis B virus (for example Hepatitis B Surface antigen or a derivative thereof), hepatitis A virus, hepatitis C virus and hepatitis E virus, or from other viral pathogens, such as paramyxoviruses: Respiratory Syncytial virus (such as F and G proteins or derivatives thereof), parainfluenza virus, measles virus, mumps virus, human papilloma viruses (for example HPV6, 11, 16, 18, such as L1, L2, E6 or E7 antigens), flaviviruses (e.g. Yellow Fever Virus, Dengue Virus, Tick-borne encephalitis virus, Japanese Encephalitis Virus) or Influenza virus, or derived from bacterial pathogens such as *Neisseria spp*, including *N. gonorrhea* and *N. meningitidis* (for example capsular polysaccharides and conjugates thereof, transferrin-binding proteins, lactoferrin binding proteins, PilC, adhesins); *Streptococcus spp*, including *S. pneumoniae* (for example capsular polysaccharides and

conjugates thereof, PsaA, PspA, streptolysin, choline-binding proteins), *S. pyogenes* (for example M proteins or fragments thereof, C5A protease, lipoteichoic acids), *S. agalactiae, S. mutans; Haemophilus spp, including H. influenzae type B* (for example PRP and conjugates thereof), *non typeable H. influenzae* (for example OMP26, high molecular weight adhesins, P5, P6, lipoprotein D), *H. ducreyi; Moraxella spp, including M catarrhalis, also known as Branhamella* catarrhalis (for example high and low molecular weight adhesins and invasins); *Bordetella spp, including B. pertussis* (for example pertactin, pertussis toxin or derivatives thereof, filamenteous hemagglutinin, adenylate cyclase, fimbriae), *B. parapertussis and B. bronchiseptica; Mycobacterium spp., including M. tuberculosis* (for example ESAT6, Antigen 85A, -B or -C), *M. bovis, M. leprae, M. avium, M. paratuberculosis, M. smegmatis; Legionella spp,* including *L. pneumophila; Escherichia spp,* including *enterotoxic E. coli* (for example colonization factors, heat-labile toxin or derivatives thereof, heat-stable toxin or derivatives thereof), enterohemorragic *E. coli,* enteropathogenic *E. coli* (for example shiga toxin-like toxin or derivatives thereof); *Vibrio spp,* including *V. cholera* (for example cholera toxin or derivatives thereof); *Shigella spp,* including *S. sonnei, S. dysenteriae, S. flexnerii; Yersinia spp,* including *Y.* enterocolitica (for example a Yop protein) , *Y. pestis, Y. pseudotuberculosis; Campylobacter spp,* including *C. jejuni* (for example toxins, adhesins and invasins) and *C. coli; Salmonella spp,* including *S. typhi, S. paratyphi, S. choleraesuis, S. enteritidis; Listeria spp.,* including *L. monocytogenes; Helicobacter spp,* including *H.* pylori (for example urease, catalase, vacuolating toxin); *Pseudomonas spp.* including *P. aeruginosa; Staphylococcus spp.,* including *S. aureus, S. epidermidis; Enterococcus spp.,* including *E. faecalis, E. faecium; Clostridium spp.,* including *C.* tetani (for example tetanus toxin and derivative thereof), *C. botulinum* (for example botulinum toxin and derivative thereof), *C. difficile* (for example clostridium toxins A or B and derivatives thereof); *Bacillus spp.,* including *B. anthracis* (for example botulinum toxin and derivatives thereof); *Corynebacterium spp.,* including *C. diphtheriae* (for example diphtheria toxin and derivatives thereof); *Borrelia spp.,* including *B. burgdorferi* (for example OspA, OspC, DbpA, DbpB), *B. garinii* (for example OspA,

OspC, DbpA, DbpB), *B. afzelii* (for example OspA, OspC, DbpA, DbpB), *B. andersonii* (for example OspA, OspC, DbpA, DbpB), *B. hermsii; Ehrlichia spp.*, including *E. equi* and the agent of the Human Granulocytic Ehrlichiosis; *Rickettsia spp.* including *R. rickettsii; Chlamydia spp., including C. trachomatis* (for example MOMP, heparin-binding proteins), *C. pneumoniae* (for example MOMP, heparin-binding proteins), *C. psittaci; Leptospira spp.*, including *L. interrogans; Treponema spp.*, including *T. pallidum* (for example the rare outer membrane proteins), *T. denticola, T. hyodysenteriae;* or derived from parasites such as *Plasmodium spp.*, including *P. falciparum; Toxoplasma spp.*, including *T. gondii (for example SAG2, SAG3, Tg34); Entamoeba spp.*, including *E. histolytica; Babesia spp.*, including *B. microti; Trypanosoma spp.*, including *T. cruzi; Giardia spp.*, including *G. lamblia; Leshmania spp.*, including *L. major; Pneumocystis spp.*, including *P. carinii; Trichomonas spp.*, including *T. vaginalis; Schisostoma spp.*, including *S. mansoni*, or derived from yeast such as *Candida spp.*, including *C. albicans; Cryptococcus spp.*, including *C. neoformans.*

Derivatives of Hepatitis B Surface antigen are well known in the art and include, inter alia, those PreS1, PreS2 S antigens set forth described in European Patent applications EP-A-414 374; EP-A-0304 578, and EP 198-474. In one preferred aspect the vaccine formulation of the invention comprises the HIV-1 antigen, gp120, especially when expressed in CHO cells. In a further embodiment, the vaccine formulation of the invention comprises gD2t as hereinabove defined.

In a preferred embodiment of the present invention vaccines containing the claimed adjuvant comprise the HPV viruses considered to be responsible for genital warts, (HPV 6 or HPV 11 and others), and the HPV viruses responsible for cervical cancer (HPV16, HPV18 and others). Particularly preferred forms of vaccine comprise L1 particles or capsomers, and fusion proteins comprising one or more antigens selected from the HPV 6 and HPV 11 proteins E6, E7, L1, and L2. The most preferred forms of fusion protein are: L2E7 as disclosed in GB 95 15478.7, and proteinD(1/3)-E7 disclosed in GB 9717953.5.

Vaccines of the present invention further comprise antigens derived from parasites that cause Malaria. For example, preferred antigens from *Plasmodia falciparum* include RTS,S and TRAP. RTS is a hybrid protein comprising substantially all the C-terminal portion of the circumsporozoite (CS) protein of *P.falciparum* linked via four amino acids of the preS2 portion of Hepatitis B surface antigen to the surface (S) antigen of hepatitis B virus. It's full structure is disclosed in the International Patent Application No. PCT/EP92/02591, published under Number WO 93/10152 claiming priority from UK patent application No.9124390.7. When expressed in yeast RTS is produced as a lipoprotein particle, and when it is co-expressed with the S antigen from HBV it produces a mixed particle known as RTS,S. TRAP antigens are described in the International Patent Application No. PCT/GB89/00895, published under WO 90/01496. A preferred embodiment of the present invention is a Malaria vaccine wherein the antigenic preparation comprises a combination of the RTS,S and TRAP antigens. Other plasmodia antigens that are likely candidates to be components of a multistage Malaria vaccine are *P. faciparum* MSP1, AMA1, MSP3, EBA, GLURP, RAP1, RAP2, Sequestrin, PfEMP1, Pf332, LSA1, LSA3, STARP, SALSA, PfEXP1, Pfs25, Pfs28, PFS27/25, Pfs16, Pfs48/45, Pfs230 and their analogues in Plasmodium spp.

The formulations may also contain an anti-tumour antigen and be useful for the immunotherapeutic treatment cancers. For example, the adjuvant formulation finds utility with tumour rejection antigens such as those for prostrate, breast, colorectal, lung, pancreatic, renal or melanoma cancers. Exemplary antigens include MAGE 1 and MAGE 3 or other MAGE antigens for the treatment of melanoma, PRAME, BAGE or GAGE (Robbins and Kawakami, 1996, Current Opinions in Immunology 8, pps 628-636; Van den Eynde et al., International Journal of Clinical & Laboratory Research (submitted 1997); Correale et al. (1997). Journal of the National Cancer Institute 89, p293. Indeed these antigens are expressed in a wide range of tumour types such as melanoma, lung carcinoma, sarcoma and bladder carcinoma. Other Tumor-Specific antigens are suitable for use with adjuvant of the present invention and include, but are not restricted to Prostate specific antigen (PSA) or Her-2/neu, KSA (GA733), MUC-1

and carcinoembryonic antigen (CEA). Accordingly in one aspect of the present invention there is provided a vaccine comprising an adjuvant composition according to the invention and a tumour rejection antigen.

One particularly preferred embodiment of the present invention is a vaccine composition comprising the hormone antigen gonadotropin releasing hormone (GnRH). Immunogenic conjugates of this antigen are disclosed in WO 95/20600, EP 0117934, US 4,302,386 and US 5,006,334. Such vaccines are especially useful in the treatment of cancer.

It is foreseen that compositions of the present invention will be used to formulate vaccines containing antigens derived from *Borrelia sp.*. For example, antigens may include nucleic acid, pathogen derived antigen or antigenic preparations, recombinantly produced protein or peptides, and chimeric fusion proteins. In particular the antigen is OspA. The OspA may be a full mature protein in a lipidated form virtue of the host cell (E.Coli) termed (Lipo-OspA) or a non-lipidated derivative. Such non-lipidated derivatives include the non-lipidated NS1-OspA fusion protein which has the first 81 N-terminal amino acids of the non-structural protein (NS1) of the influenza virus, and the complete OspA protein, and another, MDP-OspA is a non-lipidated form of OspA carrying 3 additional N-terminal amino acids.

Vaccines of the present invention may be used for the prophylaxis or therapy of allergy. Such vaccines would comprise allergen specific (for example Der p1) and allergen non-specific antigens (for example the stanworth decapeptide).

In a further aspect of the present invention there is provided a vaccine as herein described for use in medicine.

It is envisaged that a vaccine preparation of the present invention may be used to protect or treat a mammal susceptible to, or suffering from a disease, by means of administering said vaccine via a mucosal route, for example, an oral or intranasal route; or by a parenteral route, for example an intramuscular route.

In an embodiment of the present invention the ratio of tricaprylin:metabolisable oil, preferably squalene, would be in the order of 1:10 to 10:1, preferably from 1:5 to 5:1.

The ratio of QS21 : 3D-MPL will typically be in the order of 1 : 10 to 10 : 1; preferably 1 : 5 to 5 : 1 and often substantially 1 : 1. The preferred range for optimal synergy is 2.5:1 to 1:1 3D-MPL: QS21. Typically for human administration QS21 and 3D MPL will be present in a vaccine in the range 1 µg - 1000 µg, preferably 10 µg - 500 µg, more preferably 20-200 µg per dose, more preferably 20-100µg per dose, and most preferably 10-50 µg per dose. Typically the oil in water will comprise from 2 to 10% squalene, from 2 to 10% α-tocopherol and from 0.3 to 3% TWEEN 80. Preferably the ratio of squalene: α-tocopherol is equal or less than 1 as this provides a more stable emulsion. Span 85 may also be present at a level of 1%. In some cases it may be advantageous that the vaccines of the present invention will further contain another stabiliser.

Vaccine preparation is generally described in New Trends and Developments in Vaccines, edited by Voller et al., University Park Press, Baltimore, Maryland, U.S.A. 1978. Encapsulation within liposomes is described, for example, by Fullerton, U.S. Patent 4,235,877. Conjugation of proteins to macromolecules is disclosed, for example, by Likhite, U.S. Patent 4,372,945 and by Armor et al., U.S. Patent 4,474,757.

The amount of protein in each vaccine dose is selected as an amount which induces an immunoprotective response without significant, adverse side effects in typical vaccinees. Such amount will vary depending upon which specific immunogen is employed and how it is presented. Generally, it is expected that each dose will comprise 1-1000 µg of protein, preferably 2-100 µg, most preferably 4-40 µg. An optimal amount for a particular vaccine can be ascertained by standard studies involving observation of appropriate immune responses in subjects. Following an initial vaccination, subjects may receive one or several booster immunisation adequately spaced.

The method of producing the oil in water emulsions described in the examples comprises the mixing the oil phase with a PBS/TWEEN80™ solution followed by homogenisation using a homogenizer, it would be clear to a man skilled in the art that a method comprising passing the mixture twice through a syringe needle would be suitable for homogenising small volumes of liquid. Equally, the emulsification process in microfluidiser (M110S microfluidics machine, maximum of 50 passes, for a period of 2

minutes at maximum pressure imput of 6 bar (output pressure of about 850 bar)) could be adapted by the man skilled in the art to produce smaller or larger volumes of emulsion. This adaptation could be achieved by routine experimentation comprising the measurement of the resultant emulsion until a preparation was achieved with oil droplets of the required diameter.The formulations of the present invention maybe used for both prophylactic and therapeutic purposes.

Also provided by the present invention is a method for stabilising an oil in water emulsion, said oil in water emulsion not being stable for a period of two years, comprising the addition of a triglyceride to the oil phase. Preferably the triglyceride used in this method is trycaprylin.

Example 1, *Adjuvant formulation*

The oil in water emulsion adjuvants composed of an organic phase (α-tocopherol, tricaprylin and squalene), an aqueous phase (PBS), and one or several emulsifiers (including TWEEN 80), are manufactured in a similar manner to that described in WO 95/17210.

TWEEN 80 is dissolved in phosphate buffered saline (PBS) to give a 0.4% solution in the PBS. To provide 100 ml of emulsion 5g of DL α-tocopherol, 0-5ml squalene, and 0-5 ml tricaprylin are added slowly and then vortexed to mix thoroughly.

Whilst the oil phase is being stirred 90 ml of PBS/TWEEN 80 solution is added dropwise and mixed thoroughly. The resulting preparation is then homogenised using a homogenizer (ultrathurax type). (Alternatively, for small volumes the material may be homogenised by passing twice through a syringe needle).

The preparation is emulsified by passes through a microfluidiser (M110S microfluidics machine). The emulsion undergoes a maximum of 50 passes, for a period of 2 minutes at maximum pressure imput of 6 bar (output pressure of about 850 bar). The resulting oil droplets have a size of approximately 300-600 nm.

For details of the oil in water emulsions to be tested see table 1.

Table 1, *Formulation of SB26 emulsions optionally containing tricaprylin. for use in stability assays.*

Emulsions	α-Tocopherol (%)	Squalene (%)	Tricaprylin (%)	Tween 80 (%)
SB26	5	5	0	0.4
SB26T	5	0	5	0.4
SB26T1	5	4	1	0.4
SB26T2.5	5	2.5	2.5	0.4
SB26T4	5	1	4	0.4

Example 2, *Adjuvant stability 1.*

The stability of the adjuvant preparations described in the example above was investigated over varying periods of time and storage conditions. The initial size of the adjuvant preparations were compared with that found after storage over 8 hours at 75°C, 1 month being stored at either 37 or 45 °C, 12 months being stored at 4°C, or after accelerated decay test comprising centrifugation at 3000×g.

The average size of the oil in water droplets were measured by photon correlation spectroscopy and expressed as count rate (CR), intensity distribution, mass distribution, and average size of particle (table 5).

The addition of tricaprylin to the large particle oil in water emulsion adjuvant formulation, which had previously been unstable, renders such emulsions stable over long periods. This invention enables this potent laboratory-based adjuvant system to be used in the long term commercial environment.

Example 3. *Adjuvant stability 2.*

The adjuvant stability experiment (see example 2) was repeated over a longer period of time. For results see table 6.

The results demonstrate that the oil in water emulsions containing tricaprylin are stable over prolonged periods of time in comparison with those emulsions which do not.

Example 4, *Vaccination studies in mice using the stable oil in water emulsion adjuvant*

Groups of Balb/C mice were immunised intramuscularly on three occasions (days 0, 14, 28) with the experimental vaccines comprising the malaria antigen RTS,S, HIV gp120, oil in water emulsion, and optionally tricaprylin. SB26 oil in water emulsions that were used were prepared according to the techniques described in WO 95/17210. These emulsions had a mean oil droplet diameter of 500nm and had a lifetime of 1 month. SB62 (150nm - stable for more than 2 years) was also made according to the techniques described in WO 95/17210, and were compared to ensure that the resultant immune response was not impeded by the addition of tricaprylin.

Name	*Emulsion formulation*
SB26T1	α-tocopherol (2.5%), squalene (2%), tricaprylin (0.5%), TWEEN 80 (0.2%), QS21 (5μg), 3D-MPL (5μg).
SB26T2.5	α-tocopherol (2.5%), squalene (1.25%), tricaprylin (1.25%), TWEEN 80 (0.2%), QS21 (5μg), 3D-MPL (5μg).
SB62	SB62 (α-tocopherol (2.5%), squalene (2.5%), TWEEN 80 (2%), QS21 (5μg), 3D-MPL (5μg).

SB26T1 and SB26T2.5 have a mean particle size of about 500nm and are stable for over 2 years. 3 De-O-acylated monophosphoryl lipid A (3D-MPL) is known from GB2220 211 (Ribi). QS21 is a HPLC purified non toxic fraction of a saponin from the bark of the South American tree Quillaja Saponaria Molina and its method of its production is disclosed (as QA21) in US patent No. 5,057,540.

The results for the humoral responses 14 days after the third vaccination are given in the following table 2. Briefly, SB26T functioned as a very potent vaccine adjuvant. The responses generated were greater than those generated by an oil in water emulsion of smaller droplet diameter (SB62).

Table 2, *HBsAg specific antibody response*

Formulation	*IgG (total) titre*
RTS,S/gp120/3D-MPL/QS21/SB26T1	43004
RTS,S/gp120/3D-MPL/QS21/SB26T2.5	40287
RTS,S/gp120/3D-MPL/QS21/SB62	28465

The CTL responses in both isolated spleen cells and popliteal lymph node cells were also measured. The results are shown in figure 2. Briefly, the SB62 formulation successfully induced RTS,S specific (as measured by HBs antigen CTL specificity) CTL activity as measured 14 days post third vaccination. The two SB26T formulations also induced comparable levels of CTL activity.

Example 5, *Investigation of the isotype profile of antibodies generated by the adjuvant systems in mice.*

Samples were taken during the vaccination experiment described in example 4, 14 days after the final vaccination. The samples were assayed by commercially available standard ELISA assays to investigate the profile of IgG sub-isotypes that were generated. The results are given in figure 1 and table 3. briefly the stable emulsion adjuvants stimulated similar IgG sub-isotypes indicating strong Th1 and Th2 type immune responses.

Table 3, *HBsAg specific antibody isotype ratio*

Formulation	*IgG2a/IgG1*
RTS,S/gp120/3D-MPL/QS21/SB26T1	2.13
RTS,S/gp120/3D-MPL/QS21/SB26T2.5	1.8
RTS,S/gp120/3D-MPL/QS21/SB62	1.56

Example 6, *Vaccination studies in rhesus monkeys using the stable oil in water emulsion adjuvant*

Groups of 5 rhesus monkeys were immunised on three occasions (days 0, 28, and 84) with the vaccine formulations as given in table 4. The antigens RTS,S and gp120 were produced as previously described. The vaccines were administered intramuscularly as a bolus injection into the posterior part of the left (gp120) or the right (RTS,S) leg. The final volume of each vaccine was 0.5ml.

Table 4. *Vaccine formulations used in the rhesus monkey studies.*

Group	***Left leg***		***Right leg***	
	Antigen	*Adjuvant*	*Antigen*	*Adjuvant*
1	gp120 (100µg)	SB62, QS21, 3D-MPL	RTS,S (50µg)	SB62, QS21, 3D-MPL
2	gp120 (100µg)	SB26T, QS21, 3D-MPL	RTS,S (50µg)	SB26T, QS21, 3D-MPL

Blood samples were taken 14 days after each vaccination and were assayed for antigen specific humoral and cell mediated responses.

The antigen specific proliferation responses of the monkey peripheral blood mononuclear cells (PBMC) was assayed using the following procedure:

- Culture in quadruplicate 100 µl of ficoll isolated PBLs at a density of 2.10^6/ml (in Falcon round bottom tissue culture-treated 96 well plates) in RPMI1640 medium containing 5% FCS and antibiotics. Note that flat-bottom culture plates are used for the ELIspot assays.
- Add 100µl of protein per dilution at the desired concentration. Medium alone is included as the negative control and ConA (5µg/ml) as the positive control.
- Culture cells for 72 hours prior to the addition of 1 µCi/well 3H-thymidine.
- Add 20µl of 3H-TdR (1mCi/ml stock) diluted 20x in complete medium for 16-18h.
- Harvest cultures onto special filter plates using a plate harvester.
- Determine radioactivity in a beta-counter.

All of the cell cultures are incubated at 37°C, 7% CO2, 90% humidity.

The anti-RTS,S humoral responses (as measured using the HBs antigen) were assayed using an ELISA technique (see example 1) and the results are shown in figure 3. The anti-gp120 humoral responses were assayed using an ELISA technique (see example 1) and the results are shown in figure 4. The anti RTS,S and gp120 cell mediated responses, as measured by PBMC proliferation, are shown in figure 5.

Table 5. *Adjuvant stability (see example 2)*

Emulsion	Storage conditions	Size			
		CR	Intensity distr	Mass distr	Z av mean
SB26	Initial size	81	523(82%) 837(24%)	449(65%) 772(43%)	NA
	5 min 3000×g	NA			
	8 hr 75°C	40	398(61%) 727(51%)	451(16%) 832(90%)	NA
	1 month 37°C	43	458(61%) 745(53%)	503(91%) 865(13%)	NA
	1 month 45°C	45	425(66%) 733(49%)	512(71%) 874(44%)	NA
	12 months 4°C	Two phases present			
SB26T	Initial size	82	384	351	378
	5 min 3000×g	77	420	487	374
	8 hr 75°C	60	407	493	366
	1 month 37°C	60	347	480	309
	1 month 45°C	68	395	474	343
	12 months 4°C	54	275(55%) 503(54%)	481	307
SB26T1	Initial size	86	430	428	430
	5 min 3000×g	97	461	494	412
	8 hr 75°C	66	506	530	440
	1 month 37°C	84	434	490	381
	1 month 45°C	48	437	475	413
	12 months 4°C	42	302(44%) 546(65%)	491	331
SB26T2.5	Initial size	91	446	475	394
	5 min 3000×g	84	411	494	378
	8 hr 75°C	78	378(67%) 602(48%)	494	402
	1 month 37°C	68	394	493	335
	1 month 45°C	70	378	511	328
	12 months 4°C	57	290(42%) 497(68%)	480	311
SB26T4	Initial size	93	424	489	401
	5 min 3000×g	82	368	323	359
	8 hr 75°C	64	434	470	390
	1 month 37°C	68	385	467	339
	1 month 45°C	78	397	493	345
	12 months 4°C	52	286(54%) 594(52%)	507	336

footnotes: NA = not available

Table 6, *Adjuvant stability (see example 3)*

Emulsion	Timing	Size by PCS (nm)				
		DIL.(X)	CR	Intensity distr.	Mass distr.	Zav mean
SB26T	Initial size	2000	99	471	493	401
			97	434	486(94%) 786(10%)	393
	5 min. 3000g	4000	39	420	487	374
	8 hours at 75°C	4000	30	407	493	366
	1 month at 37°C	4000	30	347	480	309
	1 month at 45°C	4000	34	395	474	343
	12 months at 4°C	4000	54	275(55%) 503(54%)	481	307
	23 months at 4°C	4000	32	272(58%) 511(52%)	488	305
			33	272(53%) 454(60%)	484	291
	23 months at 20°C	4000	34	259(68%) 515(38%)	243(17%) 510(88%)	297
			34	257(55%) 475(54%)	233(5%) 505(96%)	287
SB26T1	Initial size	2000	86	430	428	430
	5 min. 3000g	4000	49	461	494	412
	8 hours at 75°C	4000	33	506	530	440
	1 month at 37°C	4000	42	434	490	381
	1month at 45°C	4000	24	437	475	413
	12 months at 4°C	4000	42	302(44%) 546(65%)	491	331
	23 months at 20°C	4000	36	298(65%) 541(45%)	489(98%) 795(4%)	331
			36	284(53%) 566(53%)	492(92%) 791(14%)	326
SB26T2.5	Initial size	2000	91	446	475	394
	5 min. 3000g	4000	42	320(56%) 501(44%)	494	378
	8 hours at 75°C	4000	39	378(67%) 602(48%)	494(97%) 796(5%)	402
	1 month at 37°C	4000	34	394	493	335
	1 month at 45°C	4000	35	378	511	328
	12 months at 4°C	4000	57	290(42%) 497(68%)	480	311
	23 months at 4°C	4000	39	296(69%) 491(46%)	482	318
			39	373	456	299
	23 months at 20°C	4000	36	347	441	305
			36	358	445	305
SB26T4	Initial size	2000	93	424	489	401
	5 min. 3000g	4000	41	368	323	359
	8 hours at 75°C	4000	32	434	470	391
	1 month at 37°C	4000	34	385	467	339
	1 month at 45°C	4000	39	397	493	345
	12 months at 4°C	4000	52	286(54%) 594(52%)	507	336
	23 months at 4°C	4000	40	274(64%) 524(45%)	254(12%) 522(92%)	309
			40	285(56%) 495(56%)	474	311
	23 months at 20°C	4000	37	275(66%) 475(46%)	463	300
			37	268(64%) 471(47%)	244(12%) 494(92%)	295

Claims:

1. An adjuvant composition comprising a stable oil in water emulsion where the oil droplets have an average diameter in the range 300 -600 nm.

2. An adjuvant composition comprising a stable oil in water emulsion, wherein said oil in water emulsion comprises a metabolisable oil and a triglyceride.

3. An adjuvant composition comprising a stable oil in water emulsion where the oil droplets have an average diameter in the range 300 -600 nm, said oil in water emulsion comprising a metabolisable oil and a triglyceride.

4. An adjuvant composition comprising a stable oil in water emulsion where the oil droplets have an average diameter in the range 350-550 nm, said oil in water emulsion comprising a metabolisable oil and a triglyceride.

5. An adjuvant composition as claimed any of claims 1 to 4, wherein said metabolisable oil is squalene.

6. An adjuvant composition as claimed in any one of claims 2 and 5, where said triglyceride is tricaprylin.

7. An adjuvant composition as claimed in any of the preceding claims, where the ratio of triglyceride : metabolisable oil is in the range 1:10 to 10:1.

8. An adjuvant composition as claimed in any of the preceding claims, where the ratio of triglyceride : metabolisable oil is in the range 1:5 to 5:1.

9. An adjuvant composition as claimed in any of the preceding claims where the oil in water emulsion further comprises one or more immunomodulators.

10. An adjuvant composition as claimed in claim 9, where the said immunomodulators are selected from the group comprising: QS21; 3D-MPL; and α-tocopherol.

11. An adjuvant composition as claimed in any one of claims 1 to 10, further comprising a stabiliser such as polyoxyethylene sorbitan monooleate (TWEEN80™).

12. A vaccine composition comprising an adjuvant composition as claimed in any one of claims 1 to 11, further comprising an antigen or antigenic preparation.

13. A vaccine composition as claimed in claim 12, wherein the antigen or antigenic preparation is prepared from the group comprising: Human Immunodeficiency Virus; Herpes Simplex Virus type 1; Herpes Simplex Virus type 2; Human Cytomegalovirus; Hepatitis A, B, C or E; Respiratory Syncitial Virus, Human Papilloma Virus; Influenza Virus; Salmonella; Neisseria; Borrelia; Chlamydia; Bordetella; Plasmodium and Toxoplasma.

14. A vaccine composition as claimed in claim 12, where the antigenic preparation is derived from a tumour antigen.

15. A vaccine composition as claimed in claim 12, where the antigenic preparation is derived from gonadotropin releasing hormone (GnRH).

16. A vaccine composition comprising an oil in water emulsion where the oil droplets have an average diameter in the range 300-600nm, an antigen or antigenic preparation, QS21, and 3D-MPL, wherein the oil in water emulsion comprises a metabolisable oil, tricaprylin, TWEEN80™, and α-tocopherol.

17. A method for manufacturing a vaccine as claimed in any one of claims 13 to 16, comprising admixing an oil in water emulsion, tricaprylin, 3D-MPL, QS21, and antigen or antigenic preparation.

18. The use of a preparation as substantially herein described for the manufacture of a medicament suitable for treating a human susceptible to, or suffering from a viral, bacterial, or parasitic disease.

19. The use of a preparation as substantially herein described for the manufacture of a medicament suitable for treating a human suffering from cancer.

20. A method of stabilising an oil in water emulsion, said oil in water emulsion not being stable for a period of two years, comprising the addition of a triglyceride to the oil phase of the oil in water emulsion.

21. A method as claimed in claim 20, wherein the triglyceride is tricaprylin.

Figure 1, *Isotype pattern of HBsAg-specific antibodies in mice (see example 5)*

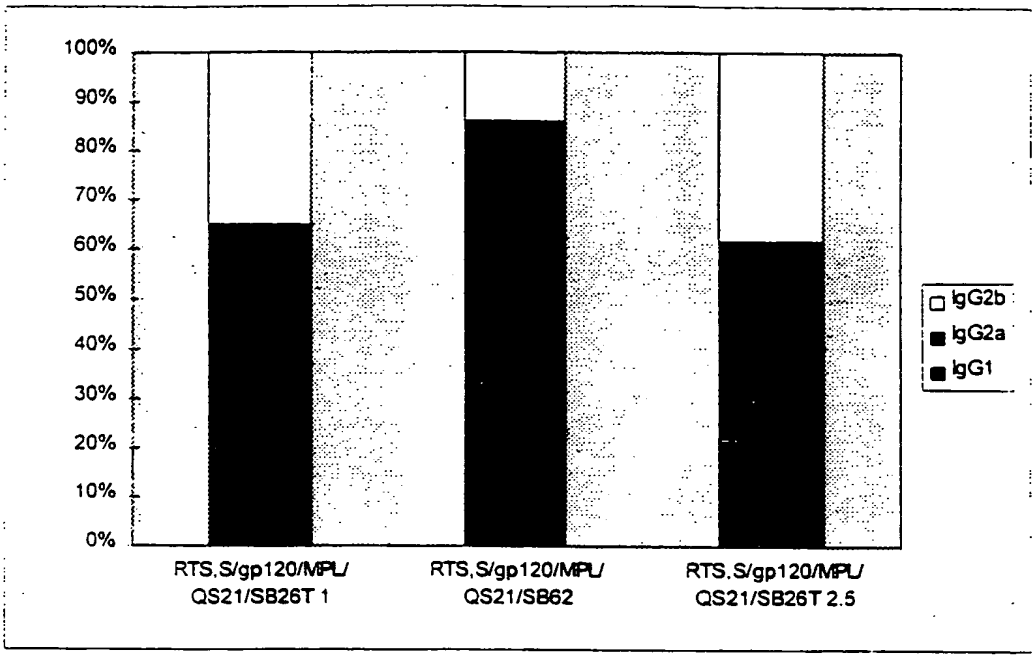


Figure 2, *HBs specific CTL activity in mouse spleen cells (see example 4)*

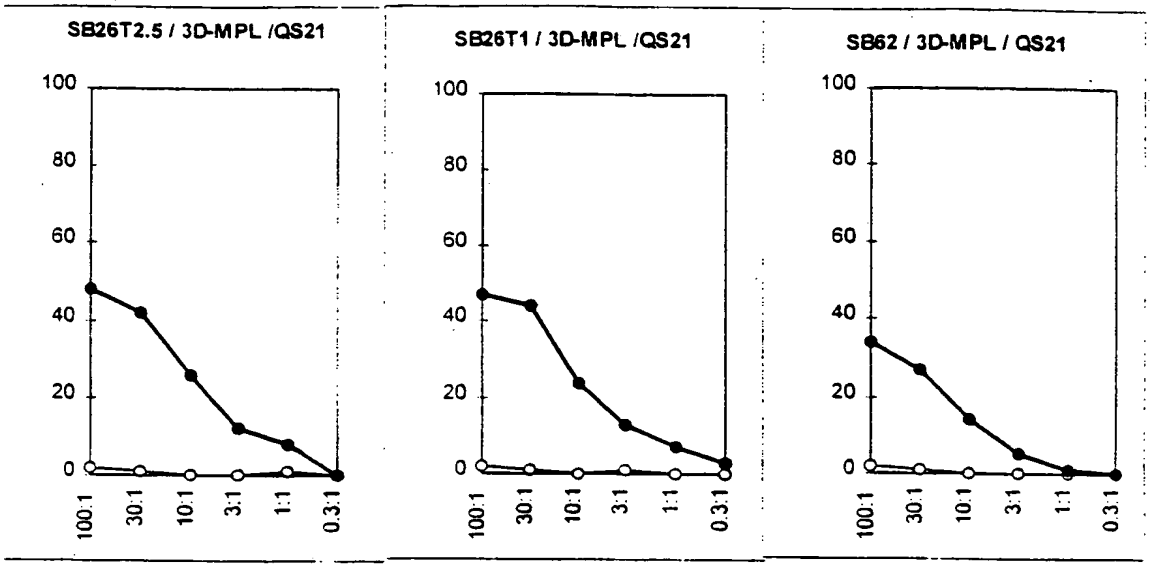


Figure 3, *RTS,S (as measured by the HBs antigen) specific antibody responses after vaccination of monkeys with the stable oil in water emulsion adjuvants. (see example 6)*

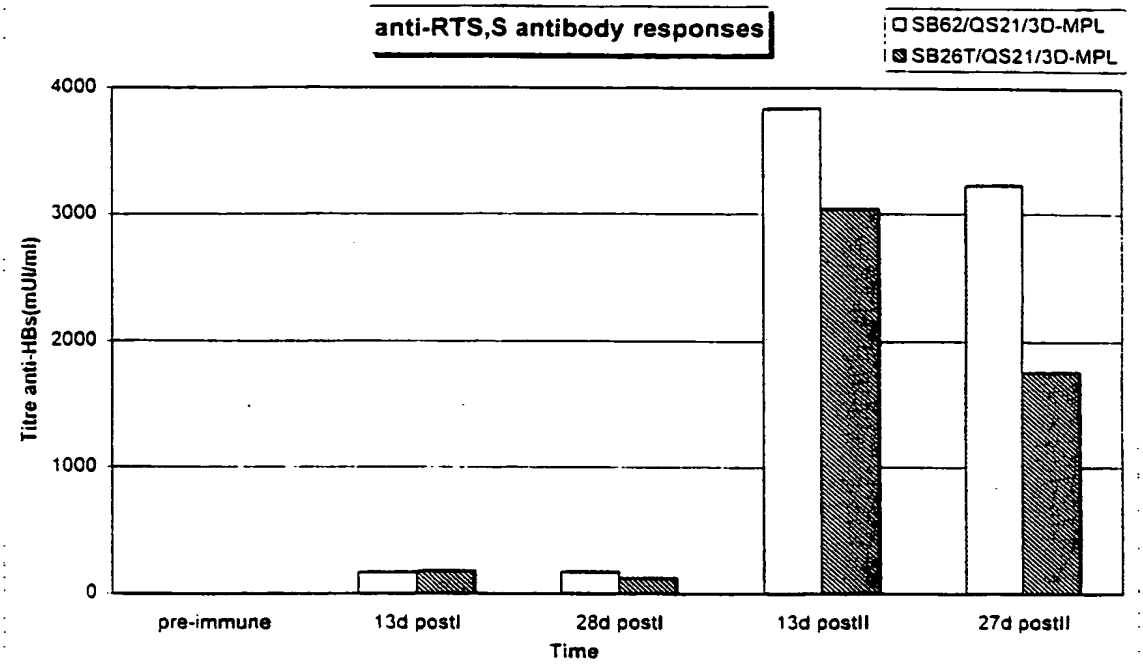


Figure 4, *gp120 specific antibody responses after vaccination of monkeys with the stable oil in water emulsion adjuvants (see example 6).*

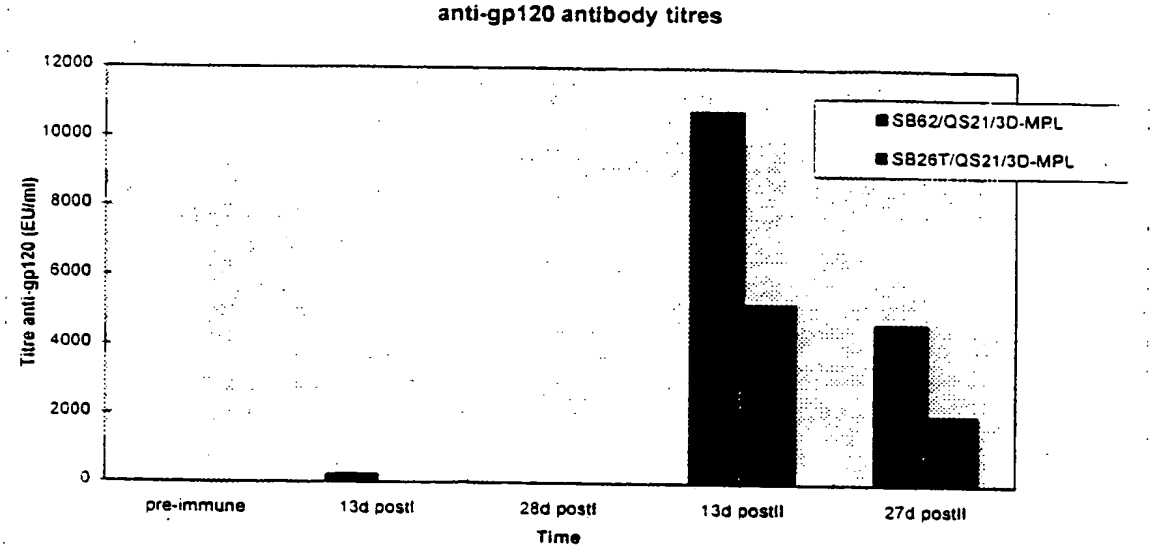
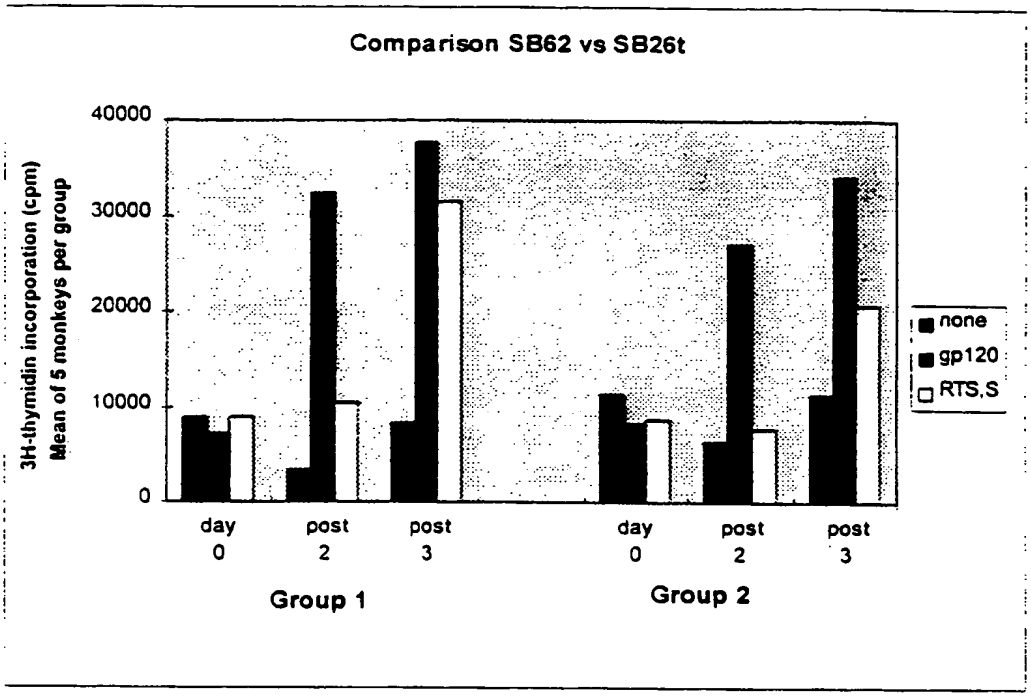


Figure 5, *PBMC proliferation after vaccination of monkeys with the stable oil in water emulsion adjuvants (see example 6)*



INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 98/03479

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K39/39 //(A61K39/39.39:21)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category '	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 11700 A (PHARMOS CORP.) 4 May 1995 see the whole document ---	1-21
X	OTT G ET AL: "Enhancement of humoral response against human influenza vaccine with the simple submicron oil / water emulsion adjuvant MF59." VACCINE, (1995 NOV) 13 (16) 1557-62, XP002081015 see the whole document --- -/--	1-21

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
16 October 1998	28/10/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Moreau, J

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INTERNATIONAL SEARCH REPORT

Inte. .onal Application No
PCT/EP 98/03479

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication where appropriate, of the relevant passages	Relevant to claim No.
A	GRANOFF D M ET AL: "MF59 adjuvant enhances antibody responses of infant baboons immunized with Haemophilus influenzae type b and Neisseria meningitidis group C oligosaccharide-CRM197 conjugate vaccine." INFECTION AND IMMUNITY, (1997 MAY) 65 (5) 1710-5, XP002081016 see the whole document ---	1-21
A	WO 95 17210 A (SMITHKLINE BEECHAM BIOLOGICALS) 29 June 1995 cited in the application see the whole document -----	1-21

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INTERNATIONAL SEARCH REPORT

Information on patent family members

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